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(54) Title: MODIFIED CELLULOSE-BINDING DOMAIN (CBD) PROTEINS AND USE THEREOF

(57) Abstract

The invention relates to modified cellulose-binding domains (CBD), and more particularly to biotinylated CBDs that show a binding affinity to cellulose similar to unmodified CBDs. Biotinylation of the CBD allows for efficient binding of biotin-binding molecules, e.g. avidin or streptavidin, to cellulose and the resultant matrix is appropriate for use as a universal affinity system. In addition, complexes of avidin or streptavidin and the biotinylated CBDs of the invention, through interaction with additional biotinylated component(s), may be used in affinity chromatography columns, diagnostic kits, enzyme reactors, drug and chemical delivery systems, and many other applications known for the avidin-biotin system in various fields of biology, biochemistry, and medicine. The invention further relates to a process for overexpression of the soluble form of CBD of the scaffoldin subunit from the cellulosome of *Clostridium thermocellum* in suitable host cells.

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MODIFIED CELLULOSE-BINDING DOMAIN (CBD) PROTEINS AND USE THEREOF

Field of the Invention

The present invention relates to modified cellulose-binding domains (CBD), and more particularly to biotinylated CBDs that show a binding affinity to cellulose similar to unmodified CBDs. Biotinylation of the CBD allows for efficient binding of avidin or streptavidin to cellulose and the resultant matrix is appropriate for use as a universal affinity system. In addition, complexes of avidin or streptavidin and the biotinylated CBDs of the invention, through interaction with additional biotinylated component(s), enjoy a wide range of applications known for the avidin-biotin complex in various fields of biology, biochemistry, medicine, etc. The invention further relates to a process for overexpression of the soluble form of CBD of the scaffoldin subunit from the cellulosome of *Clostridium thermocellum* in suitable host cells.

Background of the Invention

Cellulosomes have been identified as multienzyme complexes devoted to the efficient degradation of cellulose and hemicellulose by a highly specialized class of cellulolytic microorganisms (Bayer et al., 1994). The cellulosome concept was first described in the anaerobic cellulolytic bacterium, *Clostridium thermocellum*, the cellulase system of which was shown to comprise a discrete multifunctional, multienzyme complex which seemed to account for the efficient solubilization of insoluble cellulose by this organism (Lamed

et al., 1983). The cellulosome in *C. thermocellum* comprises numerous subunits.

In the plant, cellulose is usually coated by other polymers, i.e. xylan and lignin, which also hinder its degradation. Each of these protective polymers is degraded by different enzymes. Unlike cellulose degradation by the cellulases, xylan is degraded quite readily by the xylanases.

Cellulases and xylanases are organized into functional domains; all of them possess a catalytic domain, the sequence of which determines to which family a given enzyme belongs. Some, but not all cellulases, also have a distinct noncatalytic cellulose-binding domain (CBD). Fungal and bacterial CBDs appear to be different, the bacterial form being much larger (Ong et al., 1989).

The cellulase system of *Clostridium thermocellum* contains both cellulosomal and noncellulosomal cellulases and xylanases. Some of these have distinct CBDs. In many cases, the location and size of the CBD has been defined in the sequence of the individual cellulase. For example, the noncellulosomal *celI* has two CBDs (Hazlewood et al., 1993). The CBDs of the cellulosomal cellulase *celF* and xylanase *xynZ* have also been disclosed (Grepinet et al., 1988; Navarro et al., 1991). Cellulosomal cellulases CelE (Durrant et al., 1991) and CelS (Morag et al., 1993; Wang et al., 1993) also have CBDs but their location in the corresponding sequence has not been described.

Two CBDs have recently been described for noncatalytic proteins, called scaffoldins (Bayer et al., 1994), which are responsible for the integration of enzyme subunits (e.g., cellulases, xylanases, etc.) into the cellulosome complex.

These CBDs are thus distinct from those which are portions of cellulase genes.

The genes of two similar noncatalytic subunits of the cellulase systems of two different cellulolytic bacteria, *C. thermocellum* and *C. cellulovorans*, *cip*A *and cbp*A, respectively, have recently been sequenced (Gerngross et al., 1993; Shoseyov et al., 1992). Both genes encode for large polypeptides of similar size (ca. 1800 amino acid residues). Both have a single CBD which bears striking homology to CBDs in other bacterial cellulases. Both sequences have nine distinct but closely related domains - the cohesins - which evidently interact with the other (catalytic) subunits to form the cohesive cellulosome structure. A portion of the scaffoldin gene, termed *cip*B, has also been cloned and sequenced (Poole et al., 1992), which contains a CBD which is almost identical to that of *cip*A. The various subunits of other cellulosomes from other cellulolytic bacteria are being sequenced (Fierobe et al., 1993; Fujino et al., 1993), and it is expected that new types of CBD-bearing scaffoldins will emerge from these studies.

CBDs bind to, and are eluted from, cellulose under mild conditions and specific reagents are not required. The CBDs retain their cellulose-binding properties when fused to heterologous proteins. They were proposed as affinity tags for protein purification and for enzyme immobilization (Ong et al., 1989a). In addition, a fusion protein which comprises streptavidin with a CBD from the bacterium *Cellulomonas fimi*, was produced in the cytoplasm of *E. coli*, where it formed inclusion bodies, and was used to immobilize biotinylated enzymes in a cellulose substrate (Le et al., 1994). However, this approach has many drawbacks. For example, it is first

necessary to prepare the fusion protein by recombinant DNA methods, and the streptavidin-CBD fusion protein has to be renatured from the inclusion bodies; the yield in this case is very low (about 4%) and, consequently, the production cost is very high.

Other types of CBD fusion proteins have also been described for protein purification or enzyme immobilization purposes (Assouline et al., 1993; Ong et al., 1989a and 1989b; Ramirez et al., 1993).

Summary of the Invention

The present invention obviates the necessity of preparing individual fusion proteins comprising the CBD and a second protein of interest.

The present invention is based on the finding that a CBD molecule can be modified without affecting its high affinity for cellulose or chitin.

The present invention thus relates to a modified CBD having a hapten moiety linked to a CBD molecule or a fraction thereof, via one or more cysteine or lysine residues of the CBD sequence.

The hapten may be any molecule which interacts strongly and selectively ($K_a \geq 10^5 M^{-1}$) with an anti-hapten molecule. In a preferred embodiment, the hapten exhibits an extremely strong interaction ($K_a \equiv 10^{15} M^{-1}$) with an avidin or streptavidin molecule and is a moiety selected from the group comprising biotin, homologs, analogs and derivatives thereof.

The CBD may be derived from any cellulose- or xylan-degrading CBD-containing microorganism, preferably from *C. thermocellum*.

When the CBD has no native cysteine residue, the cysteine residue may be introduced in the CBD molecule by recombinant DNA methods.

The invention further relates to a complex comprising a hapten-modified CBD of the invention and an anti-hapten substance, for example, a complex comprising a biotinylated CBD and a biotin-binding molecule, such as avidin, streptavidin, modified avidin, modified streptavidin or antibiotin antibodies, or a conjugate of any one of the said biotin-binding molecules covalently linked to a component of a target:binder pair as defined hereinafter.

In another embodiment, the invention relates to a matrix comprising a substrate selected from chitin, cellulose or a cellulose-containing substrate, to which is bound a modified CBD or a complex thereof according to the invention. In a preferred embodiment, the modified CBD is biotinylated CBD and the complex thereof is with avidin or streptavidin.

The invention further relates to the use of the modified CBDs and of the complexes thereof in several useful applications, for example, affinity chromatography, cell separation, cell immobilization, protein and enzyme immobilization, selective removal of biological materials, multienzyme reactors, signal immunoassay and drug delivery.

Description of the Drawings

Figs. 1 A-C depict subcloning and expression of the CBD gene fragment. Fig. 1A is a schematic description of the organization of the *cipB* gene from *Clostridium thermocellum* which harbors the CBD fragment;"coh" represents the cohesins, or subunit-binding domains; "domX" (domain X) represents an

unknown function; "doc" (dockers) represents reiterated sequence of the docking domain of catalytic subunits which interacts with a cohesin domain; and "*" indicates a stop. Fig. 1B depicts the sequences of the N-terminal and C-terminal primers used for polymerase chain reaction (PCR) in cloning the *cipB* gene. Fig. 1C depicts the amino acid sequence of the CBD from the scaffoldin subunit of the cellulosome from *C. thermocellum*. In the *cipA* gene, the amino acid in position 139 was found to be alanine and in the *cipB* gene, it is arginine.

Fig. 2 shows purification of the CBD from *C. thermocellum* by affinity digestion. The host cell bacterial sonicate was adsorbed to amorphous (phosphoric acid-treated) cellulose, and washed with 3M urea. Purified cellulosome was added in order to hydrolyze the cellulose resin with consequent release of the bound CBD. The residual cellulosome and CBD were then separated on a Sephacryl S-300 column. Inserts : A). SDS-PAGE of material applied to the gel filtration column. B) SDS-PAGE of the high-molecular-weight peak, showing the expected profile of the purified cellulosome components. C) SDS-PAGE of the low-molecular-weight peak, showing the purified CBD band.

Fig. 3 depicts double-reciprocal plot of CBD adsorption to microcrystalline cellulose, wherein purified CBD (open circles) or the cysteine-biotinylated CBD (S-biotinyl CBD) derivative (closed circles) was allowed to interact with the cellulose substrate. After 1h, the samples were centrifuged and the supernatant fluids were examined for protein. The results were analyzed according to Scatchard.

Figs. 4 A-C depict: 4A: a scheme for preparation of a biotinylated (B) CBD; 4B:a scheme for isolation of an antibody (Ab) by sequential application of biotinylated CBD, avidin (Av)

and biotinylated protein A on a cellulose support; and 4C: a scheme for isolation of an antibody by sequential application of biotinylated CBD and an avidin-protein A conjugate on a cellulose support.

Fig. 5 shows direct isolation of anti-transferrin antibodies from rabbit serum by sequential application of S-biotinyl CBD, avidin, and biotinyl protein A on a cellulose support. Lanes:1 - whole serum; 2 - purified CBD; 3 - immuno-globulin G purified by the procedure of the invention; 4 - immuno- globulin G purified by conventional affinity chromatography (on a CNBr-activated Sepharose-protein A column).

Detailed Description of the Invention

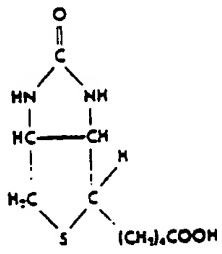
The present invention, in one of its aspects, is directed to hapten-modified CBDs, particularly to biotinylated CBDs, that bind strongly to cellulose.

The term "hapten" herein refers to a molecule which interacts strongly and selectively ($K_a \geq 10^5 M^{-1}$) with an anti-hapten molecule. Examples of such haptens are biotin, analogs, homologs and derivatives thereof, fluorescein and derivatives thereof, digoxigenin and derivatives thereof, HABA [2-(4'-hydroxyazobenzene) benzoic acid] and derivatives thereof, and peptides that are biotin-mimetics and bind to avidin or streptavidin.

The hapten-modified CBD form complexes with the appropriate molecule to which the hapten strongly and selectively binds. Examples of such systems are biotin-avidin, digoxigenin-anti-digoxigenin antibodies (DIG System, available from Boehringer, Mannheim, Germany), and fluorescein-

anti-fluorescein antibodies (available from Boehringer, Mannheim, Germany).

Biotin has the formula :



"Homologs" of biotin are obtained by adding or deleting $-CH_2-$ groups from the spacer $-(CH_2)_4-$ at position 2.

"Derivatives" of biotin are obtained by modification of the -COOH group, such as for example esters and amides as described in Green, 1975; Wilchek and Bayer, 1988; and as commercially available, e.g. from Pierce Chemical Company (Rockford, Il. U.S.A.) and Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Examples of such derivatives are iodoacetyl -LC-biotin, in which the carboxyl group is replaced by $-CO-NH-(CH_2)_6-NH-CO-CH_2I$; biocytin, in which the carboxyl group is replaced by $-CO-NH-(CH_2)_4-CH(NH_2)-COOH$; maleimido derivatives of biotin, such as maleimido-alkanoylbiocytin, more particularly maleimido-propionylbiocytin; and esters such as biotinyl N-hydroxysuccinimide or p-nitrophenyl ester. Other derivatives include substitution of the oxo radical at position 2' by a thio or imino group.

"Analogs" of biotin are, for example, the desthiobiotin (imidazolidone) and oxazolidone derivatives described by Green, 1975, pp. 106-107.

The term "CBD" or "CBD protein" as used herein refers to a cellulose-binding domain derived from a cellulose- or

xylan-degrading CBD-containing microorganism. Examples of such microorganisms include *Clostridium thermocellum*, *Clostridium cellulovorans*, *Clostridium stercorarium*, *Cauldocellum saccharolyticum*, *Clostridium cellulolyticum*, *Clostridium acetobutylicum*, *Bacillus subtilis*, *Bacillus lautus*, *Cellulomonas fimi* and *Cellulomonas uda*. A "fraction" of the CBD refers herein to any fragment, variant or chemical derivative of a CBD which binds with high affinity to cellulose. In a preferred embodiment of the invention, the CBD is derived from *C. thermocellum*, more particularly derived from the *cip*B or *cip*A gene thereof and has the amino acid sequence depicted in Fig. 1C, in which the amino acid residue X at position 139 is arginine or alanine, respectively.

The hapten moiety is linked to the CBD molecule through a cysteine or lysine residue of the CBD sequence. If the CBD has more than one cysteine or lysine residue, a hapten moiety might be linked to each of such cysteine or lysine residues.

In one preferred embodiment, a biotin moiety is linked to the CBD molecule via a cysteine residue by reaction with a sulfhydryl-specific biotinylation reagent, e.g. iodoacetyl-LC-biotin,biotin-HPDP [N-[6-(biotinamino)hexyl]-3'-(2'-pyridyldithio) propionamide], and maleimido derivatives of biotin, e.g. maleimidopropionylbiocytin and biotin-BMCC[N-[4-(biotin-amino) propyl]-4'-(maleimidomethyl) cyclohexanamide].

Many CBDs are characterized by a single homologous cysteine, enabling incorporation of a single biotin moiety using an appropriate sulfhydryl-specific reagent. Thus, CBDs have been described from *C. thermocellum*, *C. cellulovorans*, *C. stercorarium* and *Bacillus subtilis*, which possess a single cysteine residue at the identical (aligned) position. The CBD

from the CenB cellulase of *Cellulomonas fimi* has two cysteine residues at different positions. In contrast, CBDs from *Bacillus lautus* and *Cauldocellum saccharolyticum* lack cysteine residues.

In cases where the CBD has no native cysteine residue or the sulfhydryl from cysteine is not available for derivatization (e.g. where the cysteines participate in disulfide bonds or are unexposed or buried), a cysteine residue may be introduced in the CBD molecule by recombinant DNA methods, either by substituting a non-essential amino acid in the CBD sequence by a cysteine residue, or by adding a cysteine residue preferably at or near the N- or C-termini of the CBD.

In another embodiment, a biotin moiety is linked to the CBD via a lysine residue by reaction with a biotinylation reagent that reacts with primary amines, such as esters of biotin, e.g., p-nitrophenyl, N-hydroxysuccinimide and N-hydroxysulfosuccinimide esters of biotin. This is an efficient means to incorporate multiple copies of a ligand into a protein, since most proteins usually have many exposed lysine residues.

The present invention further relates to a complex comprising a hapten-modified CBD of the invention and a molecule to which said hapten strongly and selectively binds. Examples of such complexes are those comprising digoxigenin-CBD and anti-digoxigen antibodies, fluorescein-CBD and anti-fluorescein antibodies, biotin-CBD and avidin or streptavidin, and biotin-CBD and antibiotin antibodies. The terms "digoxigenin", "fluorescein" and "biotin" herein include also analogs and derivatives thereof.

The terms "avidin" and "streptavidin" as used herein include both native and modified proteins. Examples of modified avidins are N-acyl avidins, such as N-acetyl, N-succinyl and N-phthalyl avidins, obtained by reacting avidin with the suitable acid anhydride, and deglycosylated forms of avidin, such as those available under the trade names NeutrAvidin™ (Pierce Chemical Co.) and Neutralite™ Avidin (Belovo Chemicals, Bastogne, Belgium).

According to another aspect of the invention, the CBD of the cellulosomal scaffoldin subunit from *C. thermocellum* is cloned in an expression vector, such as a T7 RNA polymerase plasmid, e.g. the commercially available pET3d, pET9 and pTrc99A plasmids, and the resulting plasmid-CBD recombinant DNA is allowed to undergo overexpression of the desired CBD in soluble form at very high levels in suitable prokaryotic host cells, e.g. *E. coli* cells. The expressed CBD is then efficiently purified by a modification of a novel procedure termed affinity digestion, described in Example 2 hereinafter, which comprises adsorbing a sample containing the soluble expressed CBD to a cellulosic matrix, followed by enzymatic degradation by the cellulosome, and separation of the resulting mixture of low-molecular-weight CBD and high-molecular-weight cellulosome in the the liquid phase, e.g. by gel filtration. Sequencing of the purified protein showed the 167-amino acid sequence depicted in Fig. 1C, wherein X is arginine (R), representing a protein of molecular weight of about 18,457 dalton.

Previously, a fragment of the gene for the cellulosomal scaffoldin from *C. thermocellum* had been cloned into pBluescript SK- and expressed in a suitable *E. coli* host (Poole

et al., 1992). This fragment, termed *cip*B, exhibits a multiplicity of functional domains (Fig. 1A), one of which is a CBD. In said work, a portion of *cip*B, which included both the putative CBD and a linker sequence, was subcloned and expressed at low product levels. The resulting expressed polypeptide had a molecular weight of about 27,000 dalton.

When using the plasmid employed by Poole et al., 1992, the CBD of the present invention was also expressed at very low levels (not shown). The fact that use of a n expression vector such as the plasmid pET3d allowed expression of the CBD in the soluble form, and not in inclusion bodies, like the expression of many cloned proteins, and at very high levels (2.2 g/l), is completely unexpected. Moreover, according to Poole et al., 1992, the expressed protein had relatively large flanking regions (which accounted for about 50% of the mass), while the protein expressed according to the present invention represents the portion of the gene considered to form the actual cellulose-binding domain.

The expressed CBD was purified by affinity digestion and sequenced, and shown to present only one (non-essential) cysteine residue at position 62 (Fig. 1C). It was biotinylated at this position and both the unmodified and the S-biotinyl CBD were shown to bind to cellulose with high affinity (Kd of about 0.4 μM corresponding to $K_a = 2.5 \times 10^6 M^{-1}$.

The hapten-CBD, e.g. biotinyl-CBD, of the invention and the complexes thereof as herein defined can be bound to a chitin or cellulose substrate. Thus, the biotinylated-CBD of the invention was used to couple tetrameric avidin and streptavidin to a cellulosic substrate.

A "cellulose substrate" as used herein includes microcrystalline and amorphous cellulose, processed cellulosic products such as tissue paper, filter paper, cloth and other products which can be nearly 100% cellulose, and cellulose-containing substrates which contain less than 100% cellulose, e.g. cotton, wood, straw. Cellulosic resins that do not require chemical activation of the matrix, as in the present invention, provide very inexpensive and versatile matrices for a variety of affinity-based applications, e.g. affinity chromatography, enzyme-linked assays, biosensors, blotting technology.

The biotinylated-CBD/avidin or streptavidin system of the invention may be used in all current and future possibilities of the avidin-biotin system and related technologies, these technologies being all encompassed by the present invention. Examples of such applications and of target-binder pairs applicable to avidin-biotin technology can be found in the review of Wilchek and Bayer, 1988, herein incorporated by reference, but the invention is not limited to such applications.

Thus, target:binder pairs applicable to avidin-biotin technology are applicable also to the avidin-biotinylated CBD system of the invention wherein one of the components is biotinylated and interacted with the biotinyl-CBD/avidin or streptavidin complex of the invention. Examples of such pairs are : (i) antigens:antibodies; (ii) antibodies:protein A; (iii) lectins:glycoconjugates; (iv) enzymes:substrates, cofactors, inhibitors, etc; (v) cations:anions; (vi) hydrophobic sites:hydrophobic groups; (vii) receptors:receptor-ligands, e.g. hormones, effectors, toxins, etc.; (viii)

membranes:liposomes; (ix) nucleic acids, genes:DNA/RNA probes; (x) nucleic acids:DNA/RNA binding proteins; (xi) phages, viruses, bacteria, subcellular organelles, cells, tissues, whole organism:a suitable component according to any of (i) to (x) above which interacts selectively with the component pair.

Alternatively, one of the members of the target:binder pair can be covalently attached to the biotin-binding molecule e.g. avidin, streptavidin, etc., and the resultant conjugate is interacted directly with the biotinyl-CBD, either in solution or already bound to a cellulosic matrix (Fig. 4C).

Thus, the modified CBDs of the invention may be used in affinity chromatography to isolate and purify a target such as those indicated in (i) to (xi) above. The subsequent interaction of the biotinylated CBDs of the invention with avidin or streptavidin provides a novel mode for binding other biologically active molecules to inexpensive cellulosic resins in a variation of the universal avidin column. Thus, the additional biotin-binding sites of avidin or streptavidin linked to the biotinylated CBD, provide another handle for binding a desired biotinylated biomolecule to the cellulose matrix, to which a desired biomolecule from a sample may bound and thus be detected or isolated.

As an example of this approach, biotinyl protein A was coupled to cellulose-bound biotinyl CBD using a modified avidin bridge, and the column thus obtained was used to isolate antitransferrin antibodies from whole rabbit serum. The performance of this affinity column was at least as efficient as that of conventional protein A affinity chromatography.

By the same procedure, other biotinylated components can be bound to the free biotin-binding sites of avidin or

streptavidin complexed with the biotinylated CBD and can thus be isolated and purified. Examples of such biotinylated components are biotinylated organisms, cells or parts thereof, and biotinylated molecules, e.g. polypeptides, proteins, enzymes, antibodies, oligo- and polysaccharides, oligo- and polynucleotides (DNA, RNA), etc.

The present invention can be appplied as a diagnostic kit for the detection and/or quantification of a substance of interest in a test sample, which substance is one of the components of a target:binder pair as described in (i) to (xi) above, said kit comprising :

(a) cellulose or chitin serving as the immobilizing substrate or matrix;

(b) a biotinylated CBD capable of binding to cellulose or chitin with high affinity;

(c) a biotin-binding molecule selected from : avidin, streptavidin, modified avidin or streptavidin, or anti-biotin antibodies;

(d) a biotinylated component being the binder counterpart of the target substance to be tested; and

(e) a detectable label.

In the kit, components (a) and (b) or (a), (b) and (c) may be combined. The biotinylated component (d) can be one member of any one of the pairs listed in (i) to (xi) above, and the target for detection or quantification will be its counterpart as listed. For example, in an immunoassay method, the biotinylated component (d) can be an antigen and the target molecule an antibody.

Alternatively, instead of components (c) and (d),the kit will comprise a biotin-binding molecule as defined in (c) above

conjugated to a component being the binder counterpart of the substance to be tested, e.g. an avidin-antigen conjugate for detection of antibodies.

The detectable label (e) includes a molecule, conjugated, complexed or derivatized to a detectable probe, which molecule recognizes a target. Such a molecule can be, for example, protein A, if, as in the above example, the target is an antibody; and the detectable probe can be an enzyme linked to the protein A and capable of catalyzing a reaction resulting in a colored product. The assay can be quantified by measuring for example, the amount of color, radiation, fluorescence, chemiluminescence, etc. produced by the enzyme, by addition of a suitable substrate, as is well known in such assays, e.g. assays using the enzymes horseradish peroxidase, luciferase, etc. and their corresponding substrates.

The above immunoassay method may be applied in both the so-called sandwich mode and the competitive mode.

As an example of such an immunoassay in the sandwich mode, the quantification of an antibody in a test sample may be carried out as follows :

(a) cellulose is the immobilizing matrix to which the (b) biotinylated CBD is bound;

(c) avidin or streptavidin binds to the biotin of the CBD;

(d) the suitable biotinylated antigen binds to the vacant binding sites on the avidin and to the target antibody molecule contained in the test sample; and

(e) a protein A-enzyme conjugate binds to the antibody and the conversion of substrate to the detectable end product is used to determine the amount of antibody in the test sample.

As an example of an immunoassay in the competitive mode, (i) a biotinylated antibody is mixed with a test sample which contains a target antibody molecule; (ii) the mixture is mixed with a protein A-enzyme conjugate; (iii) the enzyme conjugate, associated with the biotinylated antibody, is immobilized onto an avidin-biotinylated CBD-cellulose immobilizing matrix; and (iv) the amount of substrate conversion is determined and compared to antibody standards. The competitive mode can be one in which the components (i)-(iii) are added sequentially or it can be a homogeneous assay wherein the components (i)-(iii) are premixed.

The present invention can also be applied as an enzyme reactor for the efficient conversion of a substrate to a product, comprising :

(a) cellulose or chitin as an inexpensive immobilizing matrix;

(b) a biotinylated CBD capable of binding to cellulose or chitin with high affinity;

(c) a biotin-binding molecule selected from : avidin, streptavidin, modified avidin or streptavidin, or anti-biotin antibodies; and

(d) a biotinylated enzyme.

Alternatively, instead of components (c) and (d), one may use a conjugate of the biotin-binding molecule and the enzyme.

The biotinylated enzyme (d) can be replaced by a biotinylated cofactor or a biotinylated antibody which recognizes a nonessential epitope on the enzyme or any biotinylated molecule which binds to the enzyme without significantly damaging its catalytic activity. The unbiotinylated enzyme can then be attached to the column

(immobilizing matrix). Once the enzyme is immobilized, substrate can be applied and the product collected. Immobilization can lead to dramatic increases in thermal and pH stabilities of an enzyme and allows continuous conversion of substrate to product. Additionally, a group of biotinylated enzymes can be immobilized together on the same matrix as a multienzyme reactor for sequential conversion of products.

Further useful applications of the modified CBDs of the present invention include targeted delivery of a drug or a chemical to an organism having a cellulosic or chitin-containing envelope or exoskeleton selected from plants, yeast, fungi, algae, insects, arthropods and crustaceans, comprising said biotinylated drug or chemical bound via the avidin to a complex comprising the biotinylated CBD according to the invention, or the drug or chemical can be derivatized to avidin, for use as a pesticide (e.g. insecticide, herbicide, fungicide, algicide and arthropodicide) or as a pharmaceutical anti-fungal composition.

The present invention can also be applied for the selective retrieval or removal of biotinylated material from a test sample, the biotinylated material being a member of any one of the pairs listed in (i) to (xi) above, particularly, an enzyme, toxin, antibody or a cell.

The invention will now be described in more detail in the following non-limiting Examples and the accompanying drawings.

EXAMPLES

General Procedures and Materials

(a) Materials. Microcrystalline cellulose was purchased from E. Merck AG, (Darmstadt, Germany). Amorphous cellulose was prepared from microcrystalline cellulose as reported previously (Lamed et al., 1985). Maleimidopropionyl biocytin and biotin N-hydroxysuccinimide ester were prepared according to Wilchek and Bayer, 1990. Sepharose-protein A was prepared by the cyanogen bromide method using 2 mg of protein A (Sigma) per ml of resin. NeutraLite avidin was obtained from Belovo Chemicals (Bastogne, Belgium). All other chemicals and biochemicals were of the highest purity commercially available.

(b) Bacterial strains and vectors. *Escherichia coli* strains HMS174, BL21 (DE3) and BL21 (DE3)pLysS and the T7 RNA polymerase expression vector pET3d were obtained from Novagen, Madison, WI., U.S.A. *E. coli* strain XL-1 Blue was obtained from StrataGene, La Jolla, Calif., U.S.A.

(c) DNA manipulation. DNA was manipulated by standard procedures (Ausubel et al., 1992; Sambrook et al., 1989). Competent E. coli cells were prepared by using calcium chloride (Sambrook et al., 1989).

(d) Miscellaneous methods. Protein determinations were carried out according to Bradford, M. 1976, using ovalbumin as a standard. Fast protein liquid chromatography (FPLC) was carried out using a Superose 12 HR 10/30 column (Pharmacia, Uppsala, Sweden). SDS-PAGE and immunoblotting were performed according to Morag et al., 1991.

Example 1

Cloning the CBD of the cellulosome subunit S1 (from *cipB*) from *Clostridium thermocellum* YS

The CBD region of the cipB gene was deduced based on sequence homology and a functional cellulose-binding assay (Poole et al., 1992). The domain consists of 167 amino acids. To clone and express the CBD of cipB, two PCR primers were designed that flank the region. Primers were made for the appropriate sequences at the beginning and end of the CBD region, namely, DNA primers with partial homology to the C and N termini of the CBD region were synthesized. The N-terminal primer (Fig. 1B) was designed to contain an ATG translational start codon inside an NcoI restriction site (CCATGG). The translational start codon is in frame with the CBD domain and within the correct distance from the T7 polymerase promoter when cloned into the NcoI cloning site of pET3d. The C-terminal primer (Fig. 1B) was designed to contain at the end of the CBD domain a stop codon (TAG) and a *Bam*HI restriction site. The restriction sites were chosen to allow facile cohesive end ligation of the amplified CBD domain into the *Nco*I and *Bam*HI sites of the T7 polymerase expression vector pET3d. Amplification of the CBD domain via the polymerase chain reaction (PCR) was performed for 30 cycles in a 100 µl volume reaction mixture containing 20 ng of template DNA, 1 µg of each primer, 200 µM of each deoxynucleoside triphosphate and 10 µg of bovine serum albumin (BSA). The PCR cycle was of 1 min at 94°C, 2 min at 50°C and 3 min at 72°C. The reaction was carried out using 2 units of Vent DNA polymerase (New England Biolabs, Beverly, MA, U.S.A.), under the buffer conditions recommended by the manufacturer. The template DNA was a 908-bp *Hpa*I-*Pst*I

fragment containing the CBD domain from plasmid pDP1 (Poole et al., 1992). The PCR product was separated on a 0.7% agarose gel, extracted from the gel with activated glass beads (Geneclean II kit; Bio 101, La Jolla, Calif., U.S.A.) and cleaved with *Nco*I and *Bam*HI. The cleaved fragment was again separated and extracted from an agarose gel, and subsequently ligated with an *Nco*I-*Bam*HI linearized pET3d plasmid DNA. Following the amplification and cloning of the CBD region into this vector, the resulting plasmid pET3d-CBD (i.e. above ligation mixture) was used to transform competent *E. coli* HMS174 cells, capable of producing T7 polymerase upon induction with IPTG. Ampicillin-resistant colonies (positive transformants) resulting from the above transformation were isolated, their plasmid DNA (pCBD) was purified and cleaved with the aforesaid restriction enzymes to verify the plasmid construct (pET3d-CBD) carried by these transformants. From the positively verified transformants, total protein analysis (SDS-PAGE) of cell extracts from IPTG-induced and -uninduced cultures indicated that a 18,000 dalton protein is produced (results not shown).

Example 2

Purification of the CBD from *cipB* of *C. thermocellum* by affinity digestion.

Preliminary experiments were carried out for conventional affinity chromatography of the expressed CBD of Example 1 on both microcrystalline and amorphous cellulose. Indeed, it was found that the adsorption of the expressed CBD protein was very efficient, but desorption procedures led to the loss of more than half of the adsorbed material. More importantly, the final

product contained low but persistent levels of contaminating polypeptide bands.

Due to the inefficiency of conventional affinity chromatography, a modification of a relatively new procedure, termed affinity digestion, shown previously to be effective for isolation of the intact cellulosome (Morag et al., 1992), was used for the purification of the CBD of Example 1. CBD-containing samples were adsorbed to a cellulosic matrix, which was then enzymatically degraded by the cellulosome. The resultant mixture of low-molecular weight CBD and high-molecular weight cellulosome in the liquid phase was facilely separated by gel filtration.

Supernatant containing crude CBD (10 ml) of Example 1 was brought to pH 7 with Tris buffer (buffer final concentration, 50 mM). Amorphous cellulose (9 mg) was added, and the final volume was brought to 22 ml. The suspension was stirred for 1h at 23°C and centrifuged. The pellet was washed twice using 200 ml of 50 mM phosphate buffer (pH 7.4), containing 1M NaCl. The cellulose resin was washed with 50 ml of 3M urea, followed by 100 ml of 50 mM sodium acetate buffer (pH 5). The washed resin was then resuspended into 20 ml of the same buffer, and 2 mg of purified cellulosome were added. The suspension was incubated with constant stirring for 2h at 60°C and centrifuged, and the supernatant fluids were saved. The pellet was again resuspended to 20 ml and the digestion procedure was repeated using 1 mg of the cellulosome preparation. When most of the cellulosic substrate was solubilized (about 16h), the mixture was centrifuged and the supernatant was pooled with the previous sample. The sample was dialyzed against 10 mM Tris buffer, concentrated by lyophilization and applied to a Sephacryl S-300

column (1.5 by 85 cm). The column was equilibrated and eluted with 50 mM Tris buffer containing 0.05% sodium azide,

With this procedure, the cellulose resin was hydrolyzed enzymatically by the cellulosome, resulting in the release of the bound CBD. Near-complete solubilization of the cellulosic matrix was observed visually, and the product (bound CBD), together with the enzyme complex, was released into the aqueous phase. Residual cellulosome was separated from the CBD by gel filtration on a Sephacryl S-300 column. Two well separated peaks resulted (Fig. 2), the second being an essentially homogeneous preparation of the low-molecular weight CBD. The final yield of the CBD preparation was calculated to be 2.2 g per liter of cell culture.

In Fig. 2A there is depicted the SDS-PAGE of the high-molecular weight peak, showing the expected profile of the purified cellulosome components; and in Fig. 2B there is depicted the SDS-PAGE of the low-molecular weight peak, showing the purified CBD band. The inserts in Fig. 2 depict the SDS-PAGE of the material applied to the gel filtration column (hydrolyzed CBD-bound cellulose resin).

Example 3

Biotinylation of the CBD from *cipB* of *C. thermocellum*

The purified CBD of Example 1 was biotinylated using two different procedures. To biotinylate the single cysteine residue at position 62 (S-biotinylation), the procedure of Bayer et al., 1985, was used, whereby maleimidopropionyl biocytin was introduced into a solution of 5 mg of CBD at a 25-fold molar excess. For multiple biotinylation of lysine groups (N-biotinylation), biotin N-hydroxysuccinimide ester was

employed according to Bayer and Wilchek, 1990a and 1990b, using a 30-fold molar excess of reagent to protein.

The cysteine residue at position 62 of the CBD was shown to be non-essential for binding activity. The purified, recombinant CBD was modified using two different sulfhydryl-specific reagents, iodoacetate and maleimido-propionyl biocytin. In both cases, the binding activity of the S-biotinylated CBD was not significantly affected (Fig. 3). Thus, biotinylation of the single cysteine of the CBD provided us with a convenient tool to selectively mediate the attachment of other molecules to cellulose, as shown in Example 5.

Example 4

Determination of cellulose-binding capacity and dissociation constants

Samples of purified CBD of Example 1 and S-biotinylated CBD of Example 3 (between 1.5 to 10.5 µg) were added to microcentrifuge tubes containing 0.5 mg of microcrystalline cellulose in Tris-HCl buffer. The final volume of the assay mixture was 100 µl. The assay tubes were mixed by vertical rotation at 24°C for 1h. The samples were then spun in a microcentrifuge for 5 min to sediment the cellulose and adsorbed CBD. The amount of protein which remained in the supernatant fluids (free CBD) was determined colorimetrically. The amount of adsorbed CBD was determined by subtracting the amount of free CBD from the total added to the assay tube. The data were analyzed by double-reciprocal plots of $(\text{bound CBD})^{-1}$ versus $(\text{free CBD})^{-1}$ according to Goldstein et al., 1993. Experiments were performed in duplicate. The results are shown in Fig. 3 for the purified CBD (open circles) and the S-

biotinylated CBD derivative (closed circles). A linear plot resulted in both cases, indicating a single type of specific interaction between the CBD (biotinylated or non-biotinylated) and the cellulosic substrate with a dissociation constant of 0.4 μM. The relatively low dissociation constant (Kd 0.4 μM, corresponding to Ka = 2.5 x 10^6 M^{-1}) was comparable to that reported for the CBD from *C. cellulovorans*. The maximum binding capacity of the CBD to cellulose was determined to be about 10 mg (0.54 μmol) of CBD per 1g of microcrystalline cellulose.

Example 5

Estimation of capacity of binding to xylan and chitin.

Samples of the S-biotinylated CBD of Example 3 (6μg) were added to microcentrifuge tubes containing 0.5 mg of xylan or chitin. The mixture was brought to a final volume of 20 μl with Tris buffer (50 mM final concentration). The assay tubes were mixed and centrifuged as above, and the supernatant fluids were examined for biotinylated CBD by a dot-blot assay (Morag et al., 1990). The biotinylated CBD, as the unmodified CBD, bound to chitin, but failed to bind to xylan. Similar results were obtained with N-biotinylated CBD (results not shown).

Example 6

Purification of IgG from serum using S-biotinylated CBD on cellulosic matrix

The suitability of the N- and S-biotinylated CBDs as tools for attaching ligands to a solid phase was examined. The fact that this modification had only a nominal effect on the binding of the CBD to cellulose, indicated that the biotinylated

derivative could serve to mediate the attachment of avidin to cellulose. In turn, the remaining free biotin-binding sites of avidin could be used to bind biotinylated protein A. Thus, protein A, immobilized in this manner to cellulose, was examined as a means to isolate antibodies directly from whole serum, according to the scheme of Fig. 4B. In this experiment, antitransferrin immunoglobulins were isolated by the direct application of rabbit serum to the cellulosic affinity column, after a sequential application of S-biotinyl CBD, avidin, and biotinyl-protein A thereon.

A sample of S-biotinylated CBD (5 mg) was brought to 5 ml with Tris buffer, and 100 mg of microcrystalline cellulose was added. The suspension was stirred for 1h at 23°C and centrifuged, and the pellet was washed once with 50 ml of phosphate buffer. The pellet was resuspended with 5 ml of the same buffer, and 4 mg of NeutraLite avidin in 5 ml of buffer was added. The suspension was stirred as described above for 1h, washed, and resuspended again, and 1 mg of biotinylated protein A was added. Protein A was modified using biotin N-hydroxysuccinimide ester, using a 20-fold molar excess of reagent to protein. The extent of biotinylation was qualitatively determined on dot blots using an avidin-complexed enzyme assay system (Bayer et al., 1990).

The resin was stirred, and the slurry was introduced into a column. The column was washed successively with 10 ml-volumes of phosphate buffer, 100 mM Tris-HCl buffer (pH 8), 10 mM Tris buffer (pH 8), and 100 mM glycine-HCl buffer (pH 3). The column was reequilibrated with 100 mM Tris buffer and 2 ml of serum which contained the antitransferrin antibodies was applied. The column was washed with 10 ml-volumes of phosphate buffer, 100

mM Tris-HCl buffer (pH 8), 10 mM Tris buffer (pH 8), and adsorbed material (protein A-bound antibodies-IgG) was eluted with the glycine-HCl buffer. Fractions of 0.8 ml were collected. The protein was determined in each fraction, and the contents of the fractions were analyzed by SDS-PAGE.

The results are shown in Fig. 5, wherein lane 1 represents whole serum before passage through modified cellulose support; lane 2 represents purified recombinant CBD; lane 3 represents purified IgG via the above-modified cellulose support; and lane 4 represents purified IgG by conventional affinity chromatography on a CNBr-activated Sepharose-protein A column. The resultant antibodies (Lane 3) appeared to be purer than those isolated on a conventional Sepharose-protein A affinity column (Lane 4). The only visible contamination of the preparation appeared to be trace levels of CBD,which apparently leaked from the column. Avidin and protein A were not observed in the gel. The high-molecular-weight serum components, which accompanied the isolation of antibody by the standard protein A affinity column, were absent in the sample prepared by affinity chromatography on the modified cellulosic matrix.

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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(2) INFORMATION FOR SEQ ID NO:3:

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(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TGTTGATGGT ACTACACTGC CACCGGGTTC TTTACC 36

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

AATTGCCTGA TACCGGTGTA TTTGCCGGTG TGTTTGTCGG 40

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

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(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Met Ala Asn Thr Pro Val Ser Gly Asn Leu
1 5 10

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Gly Lys Glu Pro Gly Gly Ser Val Val
1 5

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 167 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

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Asn Pro Ser Asp Thr Thr Asn Ser Il Asn Pro Gln Phe Lys Val Thr
20 25 30

Asn Thr Gly Ser Ser Ala Ile Asp Leu Ser Lys Leu Thr Leu Arg Tyr
35 40 45

Tyr Tyr Thr Val Asp Gly Gln Lys Asp Gln Thr Phe Trp Cys Asp His
50 55 60

Ala Ala Ile Ile Gly Ser Asn Gly Ser Tyr Asn Gly Ile Thr Ser Asn
65 70 75 80

Val Lys Gly Thr Phe Val Lys Met Ser Ser Ser Thr Asn Asn Ala Asp
85 90 95

Thr Tyr Leu Glu Ile Ser Phe Thr Gly Gly Thr Leu Glu Pro Gly Ala
100 105 110

His Val Gln Ile Gln Gly Arg Phe Ala Lys Asn Asp Trp Ser Asn Tyr
115 120 125

Thr Gln Ser Asn Asp Tyr Ser Phe Lys Ser Xaa Ser Gln Phe Val Glu
130 135 140

Trp Asp Gln Val Thr Ala Tyr Leu Asn Gly Val Leu Val Trp Gly Lys
145 150 155 160

Glu Pro Gly Gly Ser Val Val
165

CLAIMS

1. A modified cellulose-binding domain (CBD) molecule in which the CBD molecule or a fraction thereof is linked to a hapten moiety via one or more cysteine or lysine residues.

2. A modified CBD according to claim 1, wherein the hapten moiety is selected from the group comprising biotin, homologs, analogs and derivatives thereof.

3. A modified CBD according to claim 2 being a biotinylated CBD molecule.

4. A biotinylated CBD according to claim 3 wherein the biotin moiety is linked via one or more native lysine or cysteine residues of the CBD molecule, or via a cysteine residue of the CBD introduced by recombinant DNA methods, either by substituting a non-essential amino acid in the amino acid sequence of the CBD by a cysteine residue, or by adding a cysteine residue at or near the N- or C-termini of the CBD.

5. A biotinylated CBD according to claim 4, wherein a maleimido derivative of biotin is linked to a cysteine residue of the CBD molecule.

6. A biotinylated CBD according to claim 5 wherein the derivative is maleimido-alkanoyl biocytin.

7. A biotinylated CBD according to claim 6 wherein the derivative is maleimido-propionyl biocytin.

8. A biotinylated CBD according to claim 4 wherein the biotin moiety is linked to a lysine residue of the CBD through a p-nitrophenyl or an N-hydroxysuccinimide ester of biotin, an analog, a homolog or a derivative thereof.

9. A modified CBD according to any one of claims 1 to 8, wherein the CBD molecule is derived from a cellulose- or xylan-degrading CBD-containing microorganism.

10. A modified CBD according to claim 9 wherein the microorganism is *Clostridium thermocellum, Clostridium cellulovorans, Clostridium stercorarium, Cauldocellum saccharolyticum, Clostridium cellulolyticum, Clostridium acetobutylicum, Bacillus subtilis, Bacillus lautus, Cellulomonas fimi* or *Cellulomonas uda*.

11. A modified CBD according to claim 10 wherein the CBD is derived from *Clostridium thermocellum*.

12. A modified CBD according to claim 11 wherein the CBD has an amino acid sequence comprising the sequence depicted in Fig. 1C, wherein the amino acid X at position 139 is alanine or arginine.

13. A biotinylated CBD according to claim 12 wherein the derivative maleimido-propionyl biocytin is linked to the cysteine residue at position 62 of the CBD molecule.

14. A complex comprising a biotinylated CBD according to any one of claims 2 to 13 and a biotin-binding molecule selected from the group comprising avidin, streptavidin, modified avidin, modified streptavidin, and antibiotin antibodies, or a conjugate of any one of the said biotin-binding molecule covalently linked to a component of a target:binder pair selected from (i) antigens:antibodies; (ii) antibodies:protein A; (iii) lectins:glycoconjugates; (iv) enzymes:substrates, cofactors, inhibitors; (v) cations:anions; (vi) hydrophobic sites:hydrophobic groups; (vii) receptors:receptor-ligands, e.g. hormones, effectors, toxins; (viii) membranes:liposomes; (ix) nucleic acids, genes:DNA/RNA probes; (x) nucleic

acids:DNA/RNA binding proteins ; and biotinylated cells or parts thereof selected from (xi) phages, viruses, bacteria, subcellular organelles, cells, tissues, whole organisms; a suitable component according to any of (i) to (x) above which interacts selectively with the component pair.

15. A complex according to claim 14 comprising the biotinylated CBD having the amino acid sequence depicted in Fig. 1C wherein the amino acid X at position 139 is arginine, and avidin.

16. A matrix comprising a chitin substrate to which is bound a modified CBD according to any one of claims 1 to 13 or a complex according to any one of claims 14 or 15.

17. A matrix comprising cellulose or a cellulose-containing substrate to which is bound a modified CBD according to any one of claims 1 to 13 or a complex according to any one of claims 14 or 15.

18. A cellulosic matrix according to claim 17, comprising a microcrystalline or amorphous cellulose substrate to which is bound the CBD of the amino acid sequence depicted in Fig. 1C, wherein the amino acid X at position 139 is alanine or arginine, modified by maleimido-propionyl biocytin linked to the cysteine residue at position 62 of the CBD molecule.

19. An affinity chromatography column comprising a cellulose substrate to which is bound a complex according to any one of claims 14 or 15.

20. An affinity chromatography column according to claim 19 wherein the complex comprises avidin, streptavidin, modified avidin or modified streptavidin covalently conjugated to a component, or which remaining free biotin-binding sites are

bound to moieties selected from the group comprising biotinylated cells or parts thereof and biotinylated molecules.

21. Use of an affinity chromatography column according to claim 20 for isolation of a target component, wherein a biotinylated molecule is selected from the group of target:binder pairs comprising : (i) antigens:antibodies; (ii) antibodies:protein A; (iii) lectins:glycoconjugates; (iv) enzymes:substrates, cofactors, inhibitors, etc; (v) cations:anions; (vi) hydrophobic sites:hydrophobic groups; (vii) receptors:receptor-ligands, e.g. hormones, effectors, toxins, etc.; (viii) membranes:liposomes; (ix) nucleic acids, genes:DNA/RNA probes; (x) nucleic acids:DNA/RNA binding proteins ; and biotinylated cells or parts thereof selected from (xi) phages, viruses, bacteria, subcellular organelles, cells, tissues, whole organism:a suitable component according to any of (i) to (x) above which interacts selectively with the component pair, wherein one of the components of said pair being the binder component is biotinylated and interacted with the biotinylated-CBD/avidin or streptavidin complex of the column, and is capable of binding to and thereby specifically isolating the corresponding other member of said pair being the target component, when a sample containing said target component is passed through the column.

22. The use of an affinity chromatography column according to claim 21 for isolation of cells or parts thereof that bear on their surface molecular counterparts to the biotinylated molecule on the column.

23. The use of an affinity chromatography column according to claim 21 comprising a cellulose substrate to which is bound a complex comprising avidin and the biotinylated CBD having the

amino acid sequence depicted in Fig. 1C wherein the amino acid X at position 139 is arginine, and biotinylated protein A is bound to the free biotin-binding sites of avidin, for isolating antibodies directly from whole serum.

24. A diagnostic kit for the detection and/or quantification of a substance contained in a test sample, said substance being one of the components of a target:binder pair as defined in (i) to (xi) hereinbelow, said kit comprising :

(a) a chitin or cellulosic matrix according to any one of claims 16 to 18, to which is bound a complex comprising a biotinylated-CBD and a biotin-binding molecule;

(b) a biotinylated molecule being the counterpart of the target substance to be tested, wherein said target:binder pair is selected from the group of target:binder pairs comprising (i) antigens:antibodies; (ii) antibodies:protein A; (iii) lectins:glycoconjugates; (iv) enzymes:substrates, cofactors, inhibitors, etc; (v) cations:anions; (vi) hydrophobic sites:hydrophobic groups; (vii) receptors:receptor-ligands, e.g. hormones, effectors, toxins; (viii) membranes:liposomes; (ix) nucleic acids, genes:DNA/RNA probes; (x) nucleic acids:DNA/RNA binding proteins; or a biotinylated cell or part thereof selected from (xi) phages, viruses, bacteria, subcellular organelles, cells, tissues, whole organism:a suitable component according to any of (i) to (x) above which interacts selectively with the component pair, wherein one of the components of said pair being the binder component is biotinylated and interacted with said biotinylated-CBD complex of the matrix of (a) and is capable of binding specifically to the corresponding component of said pair being the target substance present in said test sample; and

(c) a detectable label which includes a molecule that is conjugated, complexed or derivatized to a detectable probe, which molecule is capable of binding specifically to the target component of (b), said detectable probe being, for example, enzymes.

25. A diagnostic kit according to claim 24 for use in an immunoassay method to test an antibody in a sample wherein the biotinylated binder component of (b) is an antigen capable of binding to such a target antibody in the test sample; and the detectable label of (c) is protein A complexed to an enzyme, said protein A being capable of binding said antibody, and said enzyme being capable of catalyzing a reaction yielding a colored product.

26. A diagnostic kit according to claim 24 for use in an immunoassay method, wherein the biotinylated binder component of (b) is an antibody capable of binding to a target component being an antigen contained in the test sample; and the detectable label of (c) is an antibody complexed to an enzyme, said antibody being capable of binding said antigen at a site other than the site of the biotinylated antibody of (b), and said enzyme being capable of catalyzing a reaction yielding a colored product.

27. An enzyme reactor for the conversion of a substrate to a product comprising :

(a) a chitin or cellulose matrix according to any one of claims 16 to 18 to which is bound a complex comprising a biotinylated-CBD and either a biotin-binding molecule or a conjugate thereof with an enzyme; and

(b) when the complex comprises a biotin-binding molecule, either a biotinylated enzyme attached to said matrix or an

unbiotinylated enzyme attached to the matrix via any binding molecule which binds to a catalytically non-essential site on the enzyme.

28. An enzyme reactor according to claim 27, wherein two or more different enzymes catalyzing different substrate-to-product reactions are attached to the matrix.

29. A drug or chemical delivery system for the targeted delivery of said drug or chemical to an organism having a cellulosic or chitin-containing envelope or exoskeleton selected from plants, yeast, fungi, algae, insects, arthropods and crustaceans, comprising said drug or chemical in biotinylated form attached to a complex according to any one of claims 14 or 15, for use as a pesticide such as herbicide, insecticide, fungicide, algicide and arthropodicide, or for use as a pharmaceutical antifungal composition.

30. A process for overexpression of the soluble form of the CBD of the cellulosomal scaffoldin subunit from *C. thermocellum* of the amino acid sequence depicted in Fig. 1c, wherein X is an alanine or arginine residue, which comprises:

(i) cloning a DNA sequence encoding said CBD in an expression vector;

(ii) allowing the resulting plasmid-CBD recombinant DNA to undergo overexpression of the desired CBD in soluble form at very high levels in suitable prokaryotic host cells; and

(iii) purifying the soluble expressed CBD by affinity digestion, which comprises adsorbing a sample containing the expressed CBD to a cellulosic matrix, followed by enzymatic degradation by the cellulosome, and separation of the resulting mixture of low-molecular-weight CBD and high-molecular-weight

cellulosome in the the liquid phase, thus recovering the purified CBD.

31. A process according to claim 30 wherein the expression vector in step (i) is a T7 RNA polymerase plasmid, the host cells in step (ii) are *E. coli* cells, and the separation of the resulting mixture in step (iii) is carried out by gel filtration.

32. A soluble form of the CBD of the amino acid sequence depicted in Fig. 1C obtained as a purified product according to the process of claim 30 or 31.

FIG. 1A

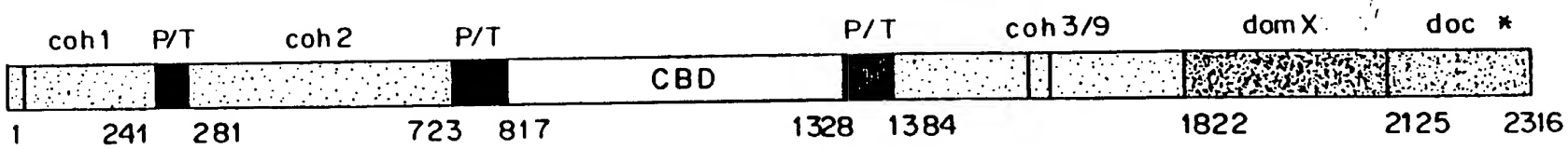


FIG. 1B

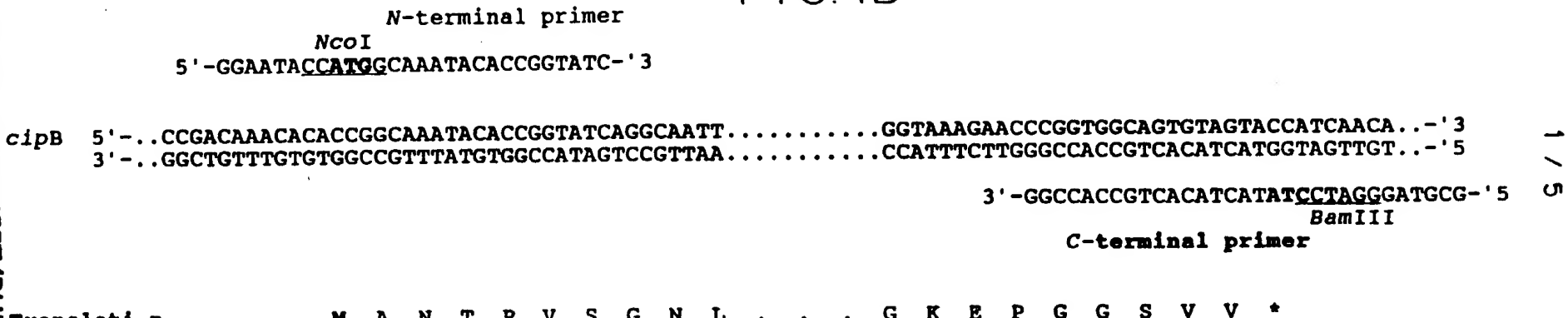


FIG. 1C

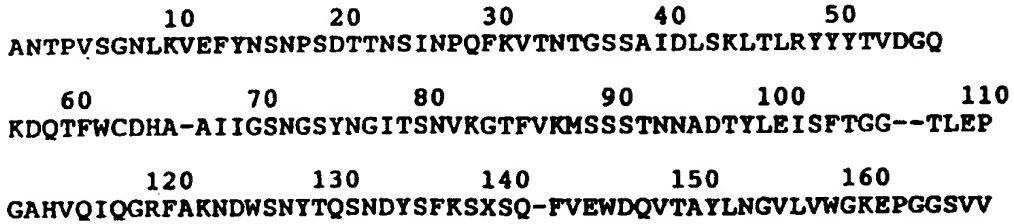


FIG. 2

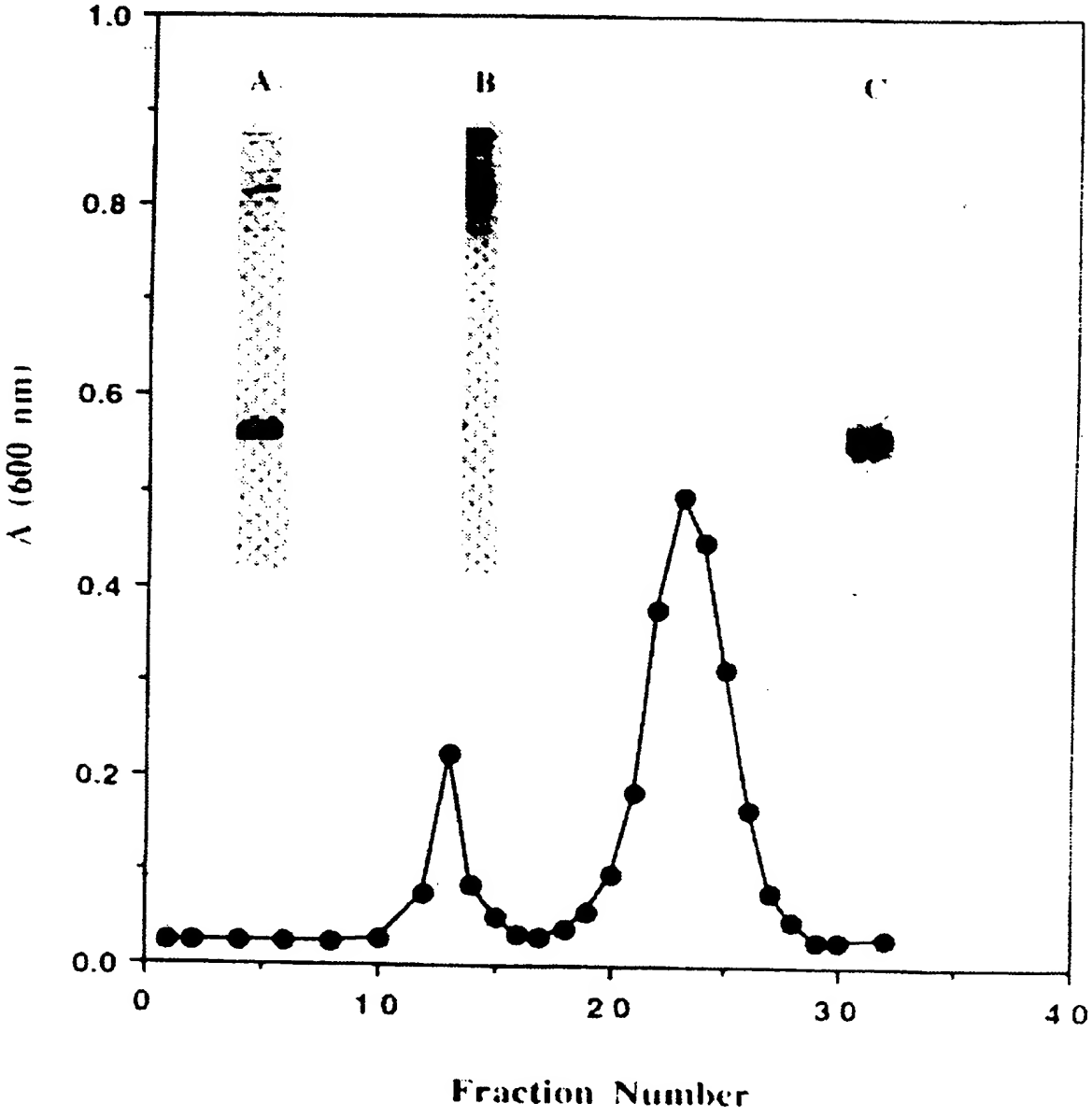


FIG. 3

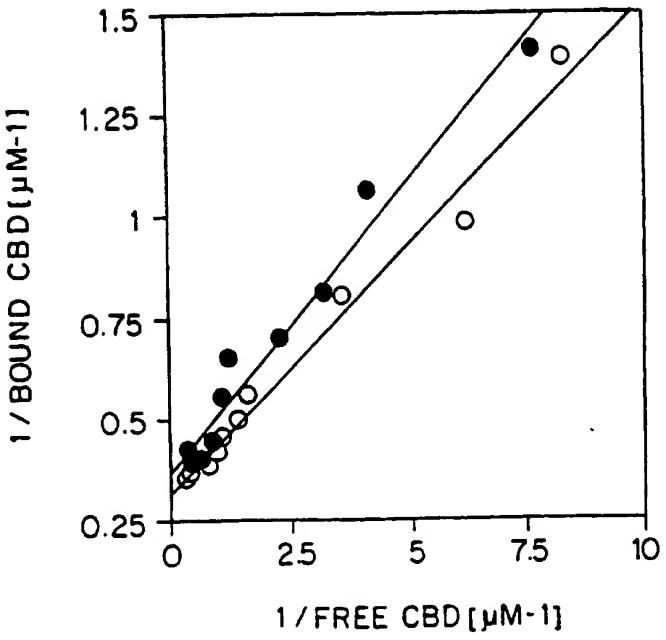


FIG. 4A

FIG. 4B

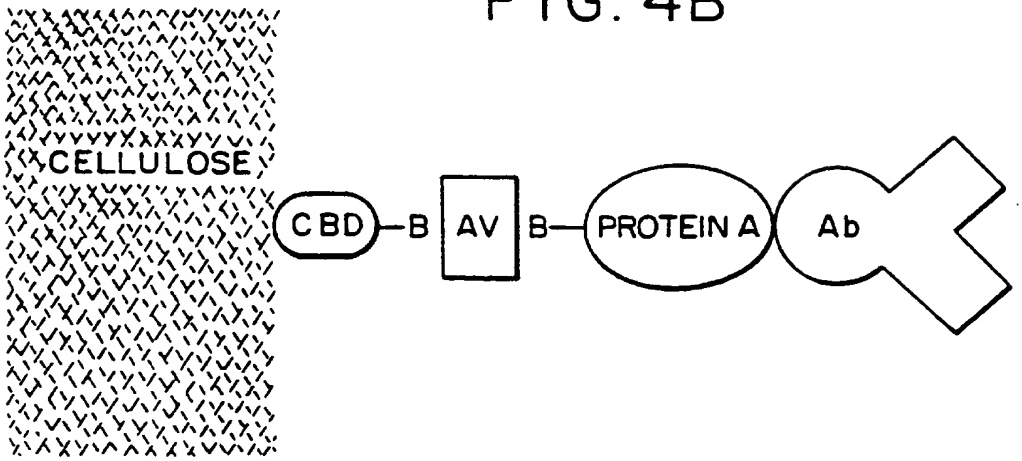


FIG. 4C

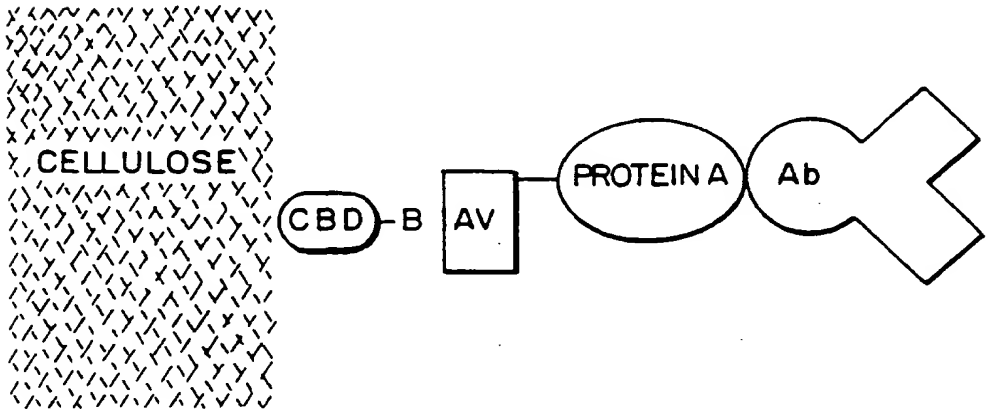
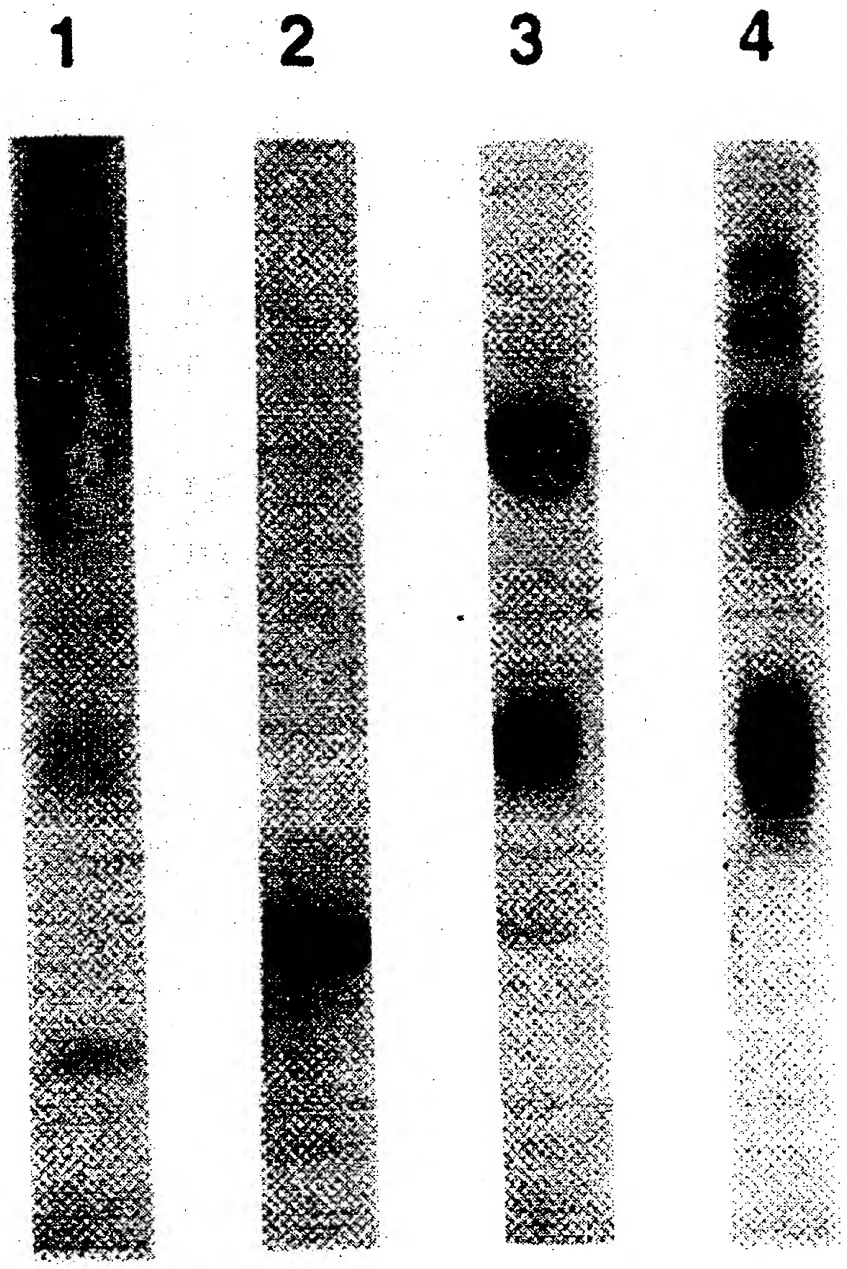


FIG.5



INTERNATIONAL SEARCH REPORT

International application No.
US95/13813

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07K 17/06; C12P 21/02; G01N 1/18, 33/544
US CL :435/69.1; 436/ 178, 530; 530/402
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1; 436/ 178, 530; 530/402

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, EMBASE
search terms: cellulose binding domain, hapten, biotin, clostridium, vector, recombinant

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 5,137,819 (KILBURN ET AL) 11 August 1992, see entire document.	1-13, 30, 31
Y	FEMS Microbiology Letters, Volume 99, issued 1992, D.M. Poole et al, "Identification of the cellulose-binding domain of the cellulosome subunit S1 from *Clostridium thermocellum* YS", pages 181-186, see entire document.	1-13, 30, 31
Y	Enzyme Microb. Technol., Volume 16, issued June 1994, K.D. Le et al, "A streptavidin-cellulose-binding domain fusion protein that binds biotinylated proteins to cellulose", pages 496-500, see entire document.	1-13, 30, 31

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
02 FEBRUARY 1996	15 FEB 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimil No. (703) 305-3230	Authorized officer NANCY PARSONS Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/13813

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BIO/TECHNOLOGY, Volume 7, issued June 1989, E. Ong et al, "Enzyme immobilization using the cellulose-binding domain of a *Cellulomonas fimi* exoglucanase", pages 604-607, see entire document.	1-13, 30, 31

Form PCT/ISA/210 (continuation of second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/13813

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
1-13, 30 and 31

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNA 'AL SEARCH REPORT

International application No.
PCT/US95/13813

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA f und multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1-13, drawn to a modified cellulose-binding domain (CBD) linked to a hapten.

Group II, claims 14 and 15, drawn to a complex of a biotinylated CBD and a biotin-binding molecule.

Group III, claims 16-23, drawn to a matrix or affinity column of substrate bound to a modified CBD or a complex of biotinylated CBD and biotin-binding molecule, and methods of using the matrix or column to isolate a target component.

Group IV, claims 24-26, drawn to a kit for detecting a target substance, the kit containing a matrix of substrate bound to a modified CBD or a complex of biotinylated CBD and biotin-binding molecule, and a biotinylated molecule which is the counterpart of the target substance.

Group V, claims 27 and 28, drawn to an enzyme reactor comprising a matrix of substrate bound to a modified CBD or a complex of biotinylated CBD and biotin-binding molecule, and a biotinylated enzyme attached to the matrix.

Group VI, claim 29, drawn to a drug or chemical delivery system comprising a biotinylated drug or chemical attached to a complex of a biotinylated CBD and a biotin-binding molecule.

Group VII, claims 30 and 31, drawn to a process or overexpression of the CBD from C. thermocellum.
Group VIII, claim 32, drawn to a soluble form of CBD.

The inventions listed as Groups I-VIII do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The complex of CBD-hapten (Group I), the complex of biotinylated CBD and biotin-binding molecule (Group II), and the matrix or column of the complex of Group II attached to a substrate (Group III) have different components and different uses and are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept, as reflected by the use of the matrix for isolating target component (Group III), the kit (Group IV) containing the matrix of Group III with a biotinylated molecule and a label, the enzyme reactor of Group V, and the drug or chemical delivery system of Group VI. The overexpression process of Group VII and the CBD product of Group VIII are not linked by a special technical feature to any of the other groups because the other groups do not specify any process of obtaining the CBD.